IFNγ-induced JAK/STAT-, but not NF-κB-, signaling pathway is insensitive to glucocorticoid in airway epithelial cells

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D.O. performed experiments and wrote manuscript; B.B. performed experiments; B.K. performed experiments; Y.A. study design and manuscript editing; A.K. performed experiments; J.D.G. performed experiments; O.T. created study design, wrote manuscript, and edited.

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Abstract

Although the majority of asthmatics are well controlled by inhaled glucocorticoids (GCs), severe asthmatics are poorly responsive to GCs. This latter group is responsible for a disproportionate share of healthcare costs associated with asthma. Recent studies in immune cells have incriminated interferon gamma (IFNγ) as a possible trigger of GC insensitivity in severe asthma; however, little is known about the role of IFNγ in modulating GC effects in other clinically relevant non-immune cells, such as airway epithelial cells. We hypothesized that IFNγ-induced JAK/STAT-associated signaling pathways in airway epithelial cells are insensitive to GCs, and that strategies aimed at inhibiting JAK/STAT pathways can restore steroid responsiveness. Using western blot analysis we found that all steps of the IFNγ-induced JAK/STAT signaling pathway were indeed GC insensitive. Transfection of cells with reporter plasmid showed IFNγ-induced STAT1-dependent gene transcription to be also GC insensitive. Interestingly, real-time PCR analysis showed that IFNγ-inducible genes (IIGs) were differentially affected by GC, with CXCL10 being GC sensitive and CXCL11 and IFIT2 being GC insensitive. Further investigation showed that the differential sensitivity of IIGs to GC was due to their variable dependency to JAK/STAT versus NF-κB signaling pathways with GC sensitive IIGs being more NF-κB-dependent and GC insensitive IIGs being more JAK/STAT-dependent. Importantly, transfection of cells with siRNA-STAT1 was able to restore steroid responsiveness of GC insensitive IIGs. Taken together, our results show the insensitivity of IFNγ-induced JAK/STAT signaling pathways to GC effects in epithelial cells, and also suggest that targeting STAT1 could restore GC responsiveness in severe asthmatics.

Keywords: asthma, glucocorticoid insensitivity, epithelial cell, inflammation, airway remodeling
Introduction

Asthma is a chronic inflammatory airway disease characterized by inflammation, airway hyper-responsiveness (AHR), and tissue remodeling (13). Symptoms include cough, wheezing, difficulty breathing, and chest tightness (26). In the United States, approximately 39.5 million people had asthma in 2011, and 3,404 asthma related deaths were reported in 2010 (7). Importantly, the healthcare costs related to asthma management reached $50.1 billion in 2007 (7) due to, but not limited to, missed work and school days, emergency department visits, hospitalization, and medical expenses (3).

While the majority of asthmatics can be treated with glucocorticoids (GCs), an estimated 5 to 10% of patients (mainly severe asthmatics) fail to properly respond to GCs and fall into the category of GC insensitive asthmatics (2). This subset of patients requires high doses of GCs in order to maintain adequate lung function. High GC doses can lead to complications/side effects such as glaucoma, osteoporosis, and diabetes (33), which significantly increased asthma related healthcare costs. In line with this, Sullivan and colleagues found that the annual number of work and school days lost, physician visits, and total costs for patients with uncontrolled asthma was significantly higher than patients with controlled asthma (37). Consistently, patients with severe persistent asthma had an average of healthcare costs of $10,121 per patient, whereas patients with mild/moderate asthma had an average of healthcare costs of $5,011 per patient (20). Collectively, these studies indicate the significant financial burden that GC insensitivity causes. This has led to an increased need to determine the mechanism(s) by which GC insensitivity occurs and, more importantly, ways to restore GC responsiveness in this subset of asthmatics.
Numerous studies reported the important role of airway epithelial cells in the pathogenesis of asthma (18). Indeed, epithelial cells are able to produce a variety of cytokines involved in airway inflammation and remodeling (39). Importantly, the role of epithelial cells as a target for GC therapy is increasing. The epithelium is the site of deposition for inhaled GCs, and as such can be exposed to higher concentrations of GCs than any other cell type in the airway, suggesting epithelial cells as key cells in which GC insensitivity may develop.

The classical Th2 paradigm suggests that asthma is predominantly driven by Th2-stimulated cytokines, such as interleukins (IL)-4 and -5 (23). More recently however, the Th1 cytokine, interferon gamma (IFNγ), has been suggested to also have a prominent role in the pathogenesis of asthma (30). IFNγ is a prominent Th1 cytokine that stimulates immune and structural cells, such as macrophages and epithelial cells, respectively, to release chemokines that are responsible for the recruitment and infiltration of inflammatory cells, and therefore amplifies the immune response (29). More specifically, IFNγ stimulates pro-inflammatory activities and prolongs eosinophil survival, which may explain its contribution to allergic inflammation (6). At the cellular level, IFNγ activates janus kinase (JAK)/signal transducers and activators of transcription (STAT)-associated signaling pathway by binding to its two receptor-subtypes, IFNγ receptor (IFNGR)-1 and -2. These receptors dimerize and activate JAK1 and JAK2 tyrosine kinases through phosphorylation. Activated JAK1 and JAK2 recruit STAT1, and trigger phosphorylation at its tyrosine 701 residue. Phosphorylated STAT1 then homodimerizes and interacts with importin-α-1 (NP-1) to translocate to the nucleus through the nuclear pore (34). Upon entering the nucleus, the homodimerized STAT1 becomes phosphorylated at serine residue 727, binds to the IFNγ-activation site (GAS), and induces the transcription of IFNγ-inducible genes (IIGs), such as IFN regulatory factor (IRF)-1, CXCL10, CXCL11, and IFIT2 (19, 34).
Interestingly, IFNγ has been found in high levels in various chronic inflammatory airway diseases. For example, Hens and colleagues showed increased expression of IFNγ in nasal secretions of asthmatics compared to control subjects (17). Similarly, an increase in IFNγ levels has been observed in patients with chronic obstructive pulmonary disease (COPD) and emphysema, two chronic inflammatory airway diseases associated with a high rate of GC insensitivity (14, 25). Importantly, IFNγ levels appear to correlate with the disease severity of chronic inflammatory airway diseases. For example, Magnan and colleagues found that IFNγ concentrations were elevated in whole blood cell supernatants of asthmatics compared to healthy subjects, and that the percentage of IFNγ-producing CD8+ T cells positively correlated with disease severity (24). In agreement with this, Abdulamir and colleagues found that as asthma severity increases, a switch from predominantly Th2 cytokines to predominantly Th1 cytokines occurs. This Th-type switch is marked by a significant increase in IFNγ in peripheral blood lymphocytes and exhibited by a decrease in the ratio of IL-4 to IFNγ in severe asthmatics (1). Similarly, peripheral blood mononuclear cells (PBMC) from COPD patients expressed a significantly higher percentage of IFNγ-producing CD8 cells compared to normal subjects, and the levels of which positively correlated with disease severity (41). Lastly, a retrospective pilot study indicated that serum IFNγ levels correlated with longitudinal decline in FEV1 in asthmatic patients over a 3-year interval (22). Together, these studies suggest a pathogenic role for IFNγ in asthma and COPD, especially in severe cases.

Even though it is evident that IFNγ plays a role in the pathogenesis of chronic inflammatory airway diseases, its involvement in, and correlation with, GC insensitivity remains highly controversial. On one hand, several studies have shown that IFNγ levels correlate with GC insensitivity. For example, Bentley and colleagues showed a persistent expression of IFNγ
mRNA in the bronchial mucosa of asthmatics despite prednisolone treatment (4). Similarly, IFNγ levels were found to be elevated in bronchoalveolar lavage (BAL) of COPD patients, and were not significantly inhibited by dexamethasone treatment compared to healthy control subjects and smoking patients (21). Other studies went further to show that IFNγ can even promote GC insensitivity. Indeed, IFNγ has been shown to significantly reduce the sensitivity of alveolar macrophages to the inhibitory effects of dexamethasone (36). In the same study, the authors found that IFNγ-induced STAT1 phosphorylation was insensitive to dexamethasone (36). Furthermore, microarray analysis of healthy human bronchial epithelial cells demonstrated that IIGs were not significantly affected by dexamethasone after 8 hours of stimulation and only partially affected after 24 hours of stimulation (29). On the other hand, other studies showed that IFNγ levels correlate with GC sensitivity. In fact, Goleva and colleagues found that while treatment of PBMCs with IL-2/IL-4 combination decreases GC receptor (GR) nuclear translocation and the expression of GC-target gene, mitogen-activated protein kinase phosphatase (MKP)-1, the addition of IFNγ antagonized these effects and restored GR nuclear translocation and MKP1 expression (11). Interestingly, Oehling and colleagues showed that PBMCs from asthmatics exhibited a significant decrease in phytohaemagglutinin (PHA)-induced IFNγ after treatment with oral prednisone (11, 28). These studies, however, did not address asthma severity when examining IFNγ levels.

In summary, in acute asthma, IFNγ has been found to be beneficial due to its protective role in suppressing the classical Th2 inflammatory response (19). However, in severe chronic inflammatory airway diseases, IFNγ plays a detrimental role by increasing the secretion of pro-inflammatory responses and by reducing GC cellular responsiveness (4, 21, 36). Therefore, the aim of this study is to determine, in airway epithelial cells, whether IFNγ-induced JAK/STAT-
associated signaling pathway is steroid sensitive, and examine the sensitivity of several IIGs to the inhibitory effects of GCs. The hypothesis is that the IFNγ-induced JAK/STAT signaling pathway is steroid insensitive and that the expression of IIGs is differentially affected by GC.
Materials and Methods

Epithelial cell culture

Human A549 lung epithelial cells were purchased from ATCC (Manassas, VA). The culture of A549 cells was performed in F12K Kaighn’s modified medium (ATCC) supplemented with 25 mM HEPES, 12 mM NaOH, 1.7 mM CaCl₂, 2 mM L-glutamine, penicillin-streptomycin, and 10% FBS. The cell cultures were incubated at 37°C in 5% CO₂.

Normal human bronchial epithelial (NHBE) cells were purchased from Lonza (Walkersville, MD). The culture of NHBE cells was performed in bronchial epithelial growth medium (BEGM) with supplement bullet kit (Lonza) according to manufacturer’s instructions. The cell cultures were incubated at 37°C in 5% CO₂.

Culture of epithelial cells in air-liquid interface

Human bronchial epithelial cells cultured at air-liquid interface (ALI) in MucilAir medium are commercially available and were purchased from Epithelix Sarl (Geneva, Switzerland). Healthy epithelial cells were obtained from a 55 year old Caucasian male with no history of smoking, no reported pathology, and negative for HIV-1 and -2 and Hepatitis B and C. Diseased epithelial cells were obtained from a 73 year old female with COPD, a history of smoking, and negative for HIV-1 and -2 and Hepatitis B and C. Patients’ were de-identified by Epithelix and therefore, use of ALI inserts does not require IRB approval. The cell cultures were incubated at 37°C in 5% CO₂ per manufacturer’s instructions.
**A549 cell transfection**

A549 cells were transfected using FuGENE 6 transfection reagent according to manufacturer’s instructions (Promega, Madison, WI). For the various transfection experiments we used 2 µg of gamma-activated sequence (GAS) luciferase reporter plasmid (Agilent, Santa Clara, CA) and 1 µg of β-galactosidase vector (to normalize transfection efficiency) (Promega). Lysates were extracted using 1x reporter lysis buffer (Promega). The activities of luciferase and β-galactosidase were evaluated using luciferase and β-galactosidase assay kits, respectively, according to the manufacturer’s instructions (Promega). The reporter luciferase activities were normalized to β-galactosidase activity and expressed as relative light unit (RLU) (38).

**Small Interfering RNA**

Cells were transfected using Cell Line Nucleofector Kit using an Amaxa Nucleofactor II device according to manufacturer’s instructions (Amaxa Biosystems, Cologne, Germany) using program X-001. A549 cells were transfected with 250 nM of small interfering RNA (siRNA) STAT1 (Sigma-Aldrich, St Louis, MO), or scrambled siRNA control (Dharmacon, Pittsburgh, PA).

**Western Blotting**

Proteins were extracted from cells using RIPA lysis buffer (Thermo Scientific, Rockford, IL) supplemented with 2 mM sodium vanadate, 10 mM sodium fluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin and 100 µM phenylmethysulfonyl fluoride (PMSF) (Sigma-Aldrich). Protein concentration was determined using a colorimetric detergent compatible protein assay (Bio-rad, Hercules, CA). Thirty µg of protein, 1x loading buffer (Life technologies, Grand Island, NY) and RIPA lysis buffer were blotted on 4-12% SDS-PAGE gel at 150V for 1.5 hours. After
electrophoresis, proteins were transferred from the gel to a 0.45 µm Whatman nitrocellulose membrane (GE Healthcare Bio-sciences, Pittsburgh, PA) in a Mini-Protean Tetra cell (Bio-rad) at 350 mA for 1.5 hours. The membranes were blocked for 1 hour using 5% milk- or 5% BSA for phosphorylated proteins in Tween tris buffer saline (TTBS) to prevent non-specific protein binding. Immunoblot analyses were then performed as described previously (Tliba et al., 2006) using anti-phospho-STAT1 (Tyr701), anti-phospho-STAT1 (Ser727), anti-total-STAT1, anti-phospho-JAK1, anti-phospho-JAK2, anti-phospho-IKKα/β (Cell Signaling, Beverly, MA), anti-total-JAK1, anti-total-JAK2, anti-IκBα (Santa Cruz Biotechnology, Dallas, TX). For normalization purposes, the membranes were stripped and re-probed with anti-GAPDH, anti-α actinin, anti-α tubulin, or anti-lamin A/C (Santa Cruz Biotechnology). A semi-quantitative measurement was then performed. To this end, the densitometry of triplicates was assessed using ImageJ image processing software (National Institute of Health, Bethesda, MD).

Cell Viability

Cell viability was determined using MTT absorbance assay purchased from Sigma-Aldrich, according to manufacturer’s instructions.

Real-time PCR

Total RNA was extracted from A549 cells using RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer’s instructions. Reverse transcription was performed in a Thermocycler (Eppendorf, Hauppauge, NY) using GoScript reverse transcription system according to manufacturer’s instructions (Promega). Real-time PCR analysis was performed on a Mastercycler Realplex2 (Eppendorf) by using a SYBR-Green kit according to manufacturer’s instructions (Applied Biosystems, Foster City, CA). Real-time PCR was performed using
predesigned primers for CXCL10, CXCL11, IFIT2, and β actin (Sigma-Aldrich). For PCR amplification, the program consisted of 60 s at 95°C for the initial denaturation period, and 40 cycles of 95°C for 1 s, 55°C for 5 s and 72°C for 20 s. The results were calculated using the comparative cycle threshold method as previously described (38).

**Immunocytochemistry**

A549 cells were fixed using 4% formaldehyde (Sigma-Aldrich) then permeabilized for 20 minutes using 0.5% Triton X-100 solution (Life technologies). The cells were then blocked using 0.5% BSA solution to prevent non-specific protein binding. Cells were incubated at 37°C for 1 hr with the primary antibody, anti-phospho-STAT1, then incubated with a secondary antibody conjugated with Alexa Fluor 488 in the dark (Life technologies). Cells were counterstained with DAPI (Life technologies) and mounted on slides using Fluoromount G (Fisher Scientific, Pittsburgh, PA). Images were obtained using a Leica fluorescent microscope (Leica Microsystems, Buffalo Grove, IL).

**Immunohistochemistry**

Epithelial ALI cells were fixed using 4% formaldehyde solution (Sigma-Aldrich) then dehydrated using increasing concentrations of ethanol (Pharmco-Aaper, Brookfield, CT). Xylem (Sigma-Aldrich) was added to the membranes and finally, the membranes were embedded in paraffin using the services of Kimmel Cancer Center Translational Research lab (Philadelphia, PA) for hematoxylin and eosin (H&E) staining.
Materials and Reagents

Tissue culture reagents were obtained from ATCC. Human recombinant (r) IFNγ was purchased from Roche Diagnostics (Indianapolis, IN). Human rIFNβ was purchased from R&D systems (Minneapolis, MN). Fluticasone propionate (FP) was obtained from Sigma-Aldrich. IKK Inhibitor III (BMS-345541) and pan-JAK inhibitor I (DBI) were purchased from Calbiochem EMD Millipore (Darmstadt, Germany).

Statistical Analysis

Data points from individual assays represent the mean values of triplicate measurements. Significant differences among groups were assessed with t test analysis. Each set of experiments was performed with a minimum of three different human A549 cell lines.
**Results**

*IFNγ, but not IFNβ-, induced JAK/STAT signaling pathway is insensitive to GC treatment in primary bronchial epithelial cells*

First we sought to determine the sensitivity of JAK and STAT1 phosphorylation to GC treatment in NHBE cells. To this end, cells were treated with IFNγ for 8 hr in the presence or absence of FP added 2 hr after, and protein expression was examined by western blot analysis. Interestingly, we found that the ability of IFNγ to induce the phosphorylation of JAK1 and JAK2 (Fig. 1A and 1B) and of STAT1 at both Tyr701 and S727 residues was not inhibited by FP (Fig. 1C and 1D). We next sought to determine if the insensitivity of STAT1 phosphorylation to GC was specific to IFNγ or could be extended to other JAK/STAT inducers, such as IFNβ (19). To this end, cells were treated with IFNβ for 8 hr, with FP added 2 hr after. As shown in Fig. 1C and D, the ability of IFNβ to induce STAT1 phosphorylation at both Y701 and S727 residues was significantly lower than that of IFNγ. Surprisingly, while STAT1 phosphorylation at Y701 and S727 was insensitive to FP treatment in IFNγ-treated cells, such phosphorylation was partially sensitive to FP treatment in IFNβ-treated cells (Fig. 1C and D). In agreement with this, JAK1 phosphorylation was insensitive to FP treatment in IFNγ-treated, but not IFNβ-treated cells (data not shown). Taken together, this data suggested that the insensitivity of JAK/STAT signaling pathway to GC effects was specific to IFNγ.

*IFNγ-induced STAT1 nuclear translocation is steroid insensitive in airway epithelial cells*

We next examined whether IFNγ-induced STAT1 nuclear translocation was affected by GC treatment. As shown in Fig. 2A, STAT1 Y701 phosphorylation was increased in the nuclear
fraction of IFNγ-treated NHBE cells. Interestingly, the addition of FP was not able to reduce
STAT1 nuclear accumulation. However, STAT1 Y701 phosphorylation was not detected in the
cytosolic fractions either in the presence or absence of IFNγ (data not shown). Similarly,
immunocytochemistry studies showed that while no Y701 phosphorylation of STAT1 was
observed in untreated A549 cells, such phosphorylation was significantly increased only in the
nucleus of IFNγ-treated A549 cells (Fig. 2B). Furthermore, the addition of FP was unable to
inhibit the increase of Y701 phosphorylation of STAT1 in the nuclear fractions. These findings
indicated that the ability of IFNγ to promote the nuclear accumulation of active STAT1 was
insensitive to steroid treatment.

*The expression of IIGs is differentially sensitive to GC in airway epithelial cells*

We next investigated whether the insensitivity of STAT1 activation to GC effects, as
shown above, was also associated with the insensitivity of STAT1-dependent gene expression to
GC. To this end, STAT1-dependent gene transcription and the expression of well-defined
STAT1-inducible genes, IFIT2, CXCL11, and CXCL10 was assessed. Interestingly, while IFNγ
treatment increased GAS-reporter activity, FP failed to significantly inhibit such induction in
both A549 and NHBE cells (Fig. 3A and 3B). Moreover, real-time PCR analyses showed that
while IFNγ treatment increased the expression of all STAT1-dependent genes examined, FP
differentially affected their expression (Fig. 4). Indeed, while the expression of IFIT2 and
CXCL11 was completely insensitive to GC treatment, the expression of CXCL10 was partially
inhibited by GC in both A549 and NHBE cells (Fig. 4A and B). Together, these results
suggested that while IFNγ-induced GAS-reporter activity was insensitive to GC effects, IIGs
were differentially sensitive.
Steroid insensitive IIGs are more STAT1-dependent

Since IFNγ-induced STAT1 activation was insensitive to GC effects, we next sought to determine whether STAT1-dependency of IIGs determines their sensitivity to GC. To this end, the effect of various concentrations of pan-JAK inhibitor, DBI, on the expression of IIGs was tested. Real-time PCR analysis showed that DBI was able to completely inhibit the expression of CXCL11 and IFIT2 in IFNγ-treated cells at 250 nM (> 97% inhibition) (Fig. 5A and B). Interestingly, at a similar concentration (250 nM), DBI was unable to completely inhibit IFNγ-induced CXCL10 expression (< 70% inhibition) (Fig. 5C). Figure 5D shows that cell viability was not significantly affected by the use of DBI. This data suggests that the steroid insensitive IIGs studied here are more STAT1-dependent than steroid sensitive IIGs.

NF-κB-dependency of IIGs determines their differential sensitivity to GC

Previous studies showed that NF-κB and STAT1 cooperatively regulate the expression of some IIGs (9, 27). Therefore, we next sought to determine whether NF-κB-dependency of IIGs determines their differential sensitivity to GC. To this end, various concentrations of NF-κB inhibitor, BMS345541, were tested. Interestingly, we found that the steroid sensitive IIG (CXCL10) was most sensitive to NF-κB inhibition (Fig. 6C). Indeed, in IFNγ-treated cells, while BMS345541 was able to reduce the expression of CXCL10 at a concentration as low as 30 nM, BMS345541 was only able to significantly reduce the expression of steroid-insensitive IIGs (CXCL11 and IFIT2) at much higher concentration of 3 μM (Fig. 6A and B). Figure 6D shows that cell viability was not significantly affected by the use of BMS345541. These results suggest that NF-κB-dependency of IIGs determines their differential sensitivity to GC, with steroid sensitive IIGs being more NF-κB-dependent.
NF-κB inhibition interferes with IFNγ-induced STAT1-dependent gene transcription, but not with STAT1 phosphorylation

Since NF-κB inhibition reduced the expression of steroid sensitive IIGs, we next sought to determine whether NF-κB inhibition interferes with IFNγ-induced STAT1 phosphorylation and –dependent gene transcription, which were shown above to be steroid insensitive (Fig. 1D-E and Fig. 3). As expected, the ability of IFNγ to induce GAS-reporter activities was completely abrogated when cells were treated with BMS345541 (Fig. 7A). Surprisingly, STAT1 phosphorylation at Y701 and S727 residues in IFNγ-treated cells was completely insensitive to BMS345541 effects (Fig. 7B and 7C). This data suggests that NF-κB modulates STAT1 activation at the nuclear/promoter level.

IFNγ induces NF-κB activation in A549 lung epithelial cells in a steroid-sensitive manner

Studies previously demonstrated the ability of IFNγ to activate NF-κB (12). However, such induction was highly cell-specific (12). Since NF-κB seems to be involved in IFNγ-induced STAT1-dependent gene transcription, we next sought to determine whether IFNγ is able to induce NF-κB activation. As shown in Fig. 8A, IFNγ induced the phosphorylation of IKKα/β as early as 5 min of treatment. Similarly, IkB degradation was observed as early as 10 min of IFNγ treatment, with complete degradation at 60 min of IFNγ treatment (Fig. 8B). Interestingly, treatment with FP decreased IKKα/β phosphorylation and IkB degradation in IFNγ-treated cells (Fig. 9A and B). These results clearly indicate the steroid sensitive induction of NF-κB by IFNγ in A549 cells.
Steroid sensitivity of IIGs can be restored by silencing STAT1

Since we here found that IFNγ-induced JAK/STAT-associated signaling pathway, but not NF-κB signaling pathway, is insensitive to GC effects, we hypothesized that inhibiting JAK/STAT signaling pathway would restore GC responsiveness. Therefore, using siRNA-STAT1 we here found that mRNA expression of steroid insensitive IIGs (CXCL11 and IFIT2) was reduced with inhibition of STAT1 (Fig. 10A and B). Importantly, when STAT1 was inhibited, these steroid insensitive IIGs became responsive to GC effects (Fig. 10A and B). When CXCL11 protein secretion was assessed, similar results were obtained (Fig. 10C). Taken together, these results indicate that steroid sensitivity of IFNγ-induced JAK/STAT signaling pathway can be restored by the inhibition of STAT1.

Epithelial cells derived from patients with COPD display higher levels of STAT1 phosphorylation

Lastly, we sought to validate these results using primary ALI human bronchial epithelial cells obtained from healthy and COPD patients. The epithelial cells cultured on ALI were treated with IFNγ for 8 hr in the presence or absence of FP added 2 hr after. As shown in Fig. 11A, IFNγ induction of STAT1 phosphorylation at the Y701 residue was significantly higher in cells obtained from COPD patients when compared to cells obtained from healthy patients (Fig. 11A, lane 6 vs 5 and lane 8 vs 7). Interestingly, IFNγ-induced STAT1 phosphorylation at Y701 was insensitive to FP treatment (Fig. 11A, lane 7 vs 5 and lane 8 vs 6).

ALI human bronchial epithelial cells used were cultured in a condition that recapitulates the pseudostratified mucociliary phenotype observed in vivo. ALI cells are physiologically relevant as they display all features of lung epithelial cells found in the human airway, including
the presence of highly motile cilia, production and secretion of mucus, and the development of stable transepithelial electrical resistance (Fig. 11B).
While the role of IFNγ in chronic inflammatory airway diseases is controversial, numerous studies have shown increased levels of IFNγ in severe asthmatics and COPD patients (14, 17, 24, 25). Interestingly, constitutive activation of STAT1 was found in lung epithelial cells obtained from asthmatic patients, resulting in higher expression levels of IIGs e.g. IRF-1 (31). While the level of IFNγ was not increased in those patients, the degree of disease severity from which the cells were obtained was not addressed (31). In the present study, we found that the levels of activated STAT1 in lung epithelial cells differentiated in ALI after IFNγ treatment were higher in COPD patients compared to control patients (Fig. 1A). We also found that IFNγ-induced JAK/STAT-associated signaling pathway was insensitive to GC actions, an insensitivity that was reversed by STAT1 inhibition.

The effect of GC on JAK/STAT1 activation varies depending on the interferon used for cell treatment. Indeed, we found here that while IFNγ-induced STAT1 phosphorylation was GC insensitive, IFNβ-induced STAT1 phosphorylation was GC sensitive (Fig. 1C and D). Similarly, GCs were found to inhibit IFNβ-induced STAT1-STAT2 activation in macrophages (10). This data indicates that GC insensitivity of the JAK/STAT-associated signaling pathway is specific to IFNγ. Interestingly, IFNγ, through the phosphorylation and the heterodimerization of JAK1 and JAK2, activates STAT1 and promotes its homodimerization, whereas IFNβ through the phosphorylation and the heterodimerization of JAK1 and TYK2, activates STAT1 and STAT2 and their subsequent heterodimerization. Therefore, the GC insensitivity of STAT1 activated by IFNγ, but not IFNβ, may lie in the mechanisms upon which JAKs are regulated. Interestingly, several studies showed that suppressors of cytokine signaling (SOCS) proteins inhibit IFN signal
transduction by binding to phosphorylated tyrosine residues on IFN receptors and JAKs via the SH2 domain and subsequently inhibit JAKs by promoting their ubiquitination (32). Interestingly, while the induction of SOCS1 by GC has been shown to mediate GC inhibitory effects on IFNβ actions (5), the involvement of SOCS1 in mediating GC effects on IFNγ actions is only transient (15). However, further studies are still needed to delineate the role of SOCS in mediating the lack of steroid responsiveness of IFNγ- versus IFNβ-associated signaling pathways.

IFNγ is a pleiotropic cytokine and able to induce several signaling pathways besides JAK/STAT pathways, such as NF-κB signaling pathway (12). Indeed, numerous studies show JAK/STAT and NF-κB pathways cooperatively induce gene expression and promote inflammatory responses (9, 27). For example, in estrogen treated murine macrophages, the activation of both JAK/STAT and NF-κB pathways was required for iNOS and NO production, and the inhibition of either pathway significantly decreased IFNγ actions (9). Furthermore, in macrophages/microglia, Nguyen and colleagues showed that NF-κB activation mediates the induction of CD40 by IFNγ (27). Consistently, we found here that IFNγ induces IKKα/β phosphorylation and IκB degradation (Fig. 8A and B). In addition, we also found that NF-κB pharmacological inhibition using BMS345541 significantly inhibited IFNγ-induced GAS-reporter activity (Fig. 7A) and the expression of IIGs (Fig. 6A-C). Interestingly, microarray analysis of human bronchial epithelial cells showed that IFNγ treatment induced TNFα expression (29). As TNFα is known to activate the NF-κB-associated signaling pathway in merely all cell types (16), it is therefore legitimate to speculate that IFNγ effects on NF-κB shown here may be due to an autocrine action of TNFα however, further studies are needed to validate such hypothesis.
Interestingly, we found that IIGs are differentially sensitive to GC effects. Indeed, while CXCL10 was sensitive to GC actions, CXCL11, and IFIT2 were insensitive (Fig. 4A and B). In agreement with this and in murine macrophages, three IIGs i.e. FcγRI, FcγRIIIα, and Ia antigen were found to be differentially affected by dexamethasone treatment (35). It is tempting to speculate that the variable sensitivity of IIGs to GC is due to their dependency to signaling pathways that also show variable sensitivity to GC. Since we here show that IFNγ not only activated JAK/STAT signaling pathway, but also NF-κB signaling pathway, we hypothesized that the differential sensitivity of IIGs to GCs is due to their variable dependencies to different signaling pathways induced by IFNγ. Indeed, we found that GC sensitive IIGs i.e. CXCL10 were more dependent on NF-κB (Fig. 6C) than the GC insensitive IIGs i.e. CXCL11, and IFIT2 (Fig. 6A and B). We also found that GC insensitive IIGs were more STAT1-dependent (Fig. 5A-C), suggesting that STAT1-dependency of IIGs determines their lack of GC responsiveness. However, additional IIGs need to be explored in epithelial and other airway cells in order to generalize such observation, as our study focused only on 3 IIGs.

Interestingly, using STAT1 Tyr701 mutant, Walter and colleagues were able to reduce STAT1 activation and subsequently the expression of IIG i.e. ICAM1 (40). Furthermore, the pharmacological inhibition of JAK/STAT1-signaling pathway in human airway smooth muscle cells has been shown to restore steroid responsiveness of GC insensitive genes (8). In agreement with this, we here show that siRNA STAT1 not only reduced the expression of all IIGs, but also increased the responsiveness of GC insensitive IIGs to GC actions (Fig 10A and B). Indeed, while GC was unable to inhibit the expression of steroid insensitive IIGs in siRNA control-transfected cells, it significantly reduced the expression of such genes in siRNA STAT1-transfected cells (Fig. 10A and B). These findings suggest that targeting STAT1 could restore...
steroid responsiveness in severe asthmatics and COPD patients where the levels of IFNγ and its target genes (IIGs) are high (4, 21, 36).

In conclusion, our study demonstrated that IFNγ-induced JAK/STAT-associated signaling pathway was insensitive to GC actions in airway epithelial cells, and that targeting STAT1 restores GC responsiveness of different IIGs. This could help in the design of novel drugs that will act as steroid-sparing agents, especially in patients requiring high doses of GC therapy. However, further studies are still needed to determine whether IFNγ-induced STAT1 activation, in turn, affects GR functions.

Grants

This work was funded by National Institutes of Health grant R01HL111541.

Disclosures

No conflicts of interest are declared by the authors.


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**Figure Captions**

**Fig. 1.** IFNγ-, but not IFNβ-, induced JAK/STAT signaling pathway is insensitive to GC effects. (A and B) NHBE cells were treated for 8 hr with IFNγ (1000 IU ml⁻¹) in the presence or absence of FP (100 nM) added 2 hr after. Protein expression of (A) phosphorylated JAK1 and (B) phosphorylated JAK2 was measured by western blot analysis. (C and D) NHBE cells were treated with IFNγ (1000 IU ml⁻¹) or IFNβ (1000 IU ml⁻¹) for 8 hr in the presence or absence of FP added 2 hr after. Protein expression of phosphorylated STAT1 at (C) Y701 residue and (D) S727 residue was measure by western blot analysis. ** P <0.01 compared to untreated cells, *** P < 0.001 compared to untreated cells. NS, non-significant compared to cells treated with IFNγ alone. # P <0.05 compared with cells treated with IFNβ.

**Fig. 2.** IFNγ-induced phosphorylated STAT1 nuclear translocation is insensitive to GC effects. (A) NHBE cells were treated with IFNγ (1000 IU ml⁻¹) for 8 hr in the presence or absence of FP (100 nM) added 2 hr after, and western blot analysis was used to examine expression of phosphorylated STAT1 at Y701 in the nuclear fraction, in which lamin A/C is used as a positive control. (B) A549 cells stimulated with IFNγ in the presence or absence of FP were fixed, permeabilized, and incubated with DAPI to stain the nucleus and anti-phosphorylated STAT1 at Y701 residue, then incubated with Alexa Fluor 488, a secondary fluorescein antibody. *** P < 0.001 compared to untreated cells.

**Fig. 3.** IFNγ-induced GAS-luciferase reporter activity is insensitive to GC effects. (A) A549 and (B) NHBE cells were cotransfected with 2 µg of GAS-luciferase reporter plasmid and 1 µg of β-galactosidase vector then treated with IFNγ (1000 IU ml⁻¹) for 8 hr in the presence or absence of FP (100 nM) added 2 hr after. Luciferase activity was measured and normalized to β-
galactosidase levels. ** P < 0.01 compared to untreated cells, *** P < 0.001 compared to untreated cells. NS, non-significant compared to cells treated with IFNγ alone.

**Fig. 4. Effects of FP on IFNγ-inducible genes’ expression.** (A) A549 and (B) NHBE cells were treated with IFNγ (1000 IU ml\(^{-1}\)) for 8 hr in the presence or absence of FP (100 nM) added 2 hr after. mRNA expression of CXCL11, IFIT2, and CXCL10 was assessed using real time PCR analysis. Real time PCR results were normalized to β actin and expressed as percent of IFNγ induction. *** P < 0.001 compared to untreated cells. NS, non-significant compared to cells treated with IFNγ alone. ## P <0.01 compared to cells treated with IFNγ alone, ### P <0.001 compared to cells treated with IFNγ alone.

**Fig. 5. Effects of pan-JAK inhibitor on IFNγ-inducible genes’ expression.** A549 cells were treated with various concentrations of DBI for 1 hr before treatment with IFNγ (1000 IU ml\(^{-1}\)) for 8 hr. mRNA expression was examined using real time PCR analysis for steroid insensitive IIGs (A) CXCL11 and (B) IFIT2, and steroid sensitive IIG (C) CXCL10. Real time PCR results were normalized to β actin and expressed as percent of IFNγ induction. (D) Cell viability was determined using MTT absorbance assay. # P <0.05, ## P <0.01, and ### P <0.001 compared to cells treated with IFNγ alone.

**Fig. 6. Effects of IKK/NF-κB inhibitor on IFNγ-inducible genes’ expression.** A549 cells were treated with various concentrations of BMS345541 for 1 hr before treatment with IFNγ (1000 IU ml\(^{-1}\)) for 8 hr. mRNA expression was examined using real time PCR analysis for steroid insensitive IIGs (A) CXCL11 and (B) IFIT2, and steroid sensitive IIG (C) CXCL10. Real time PCR results were normalized to β actin and expressed as percent of IFNγ induction. (D) Cell
viability was determined using MTT absorbance assay. # P <0.05, ## P <0.01, and ### P <0.001 compared to cells treated with IFNγ alone.

**Fig. 7.** DBI inhibits IFNγ-induced JAK/STAT pathway, whereas BMS345541 only inhibits STAT1-dependent gene transcription. A549 cells were treated with DBI (25 µM) or BMS345541 (30 µM) for 1 hr before treatment with IFNγ (1000 IU ml⁻¹) 8 hr. (A) Cells were cotransfected with 2 µg of GAS-luciferase reporter plasmid and 1 µg of β-galactosidase vector before treatment. Luciferase activity was measured and normalized to β-galactosidase levels. Protein expression of phosphorylated STAT1 at (B) Y701 residue and (C) S727 residue was measured by western blot analysis. *** P < 0.001 compared to untreated cells, ### P <0.001 compared to cells treated with IFNγ alone, NS, non-significant compared to cells treated with IFNγ alone.

**Fig. 8.** IFNγ induces NF-κB-associated signaling pathway through IKKα/β phosphorylation and IκB degradation. A549 cells were treated with IFNγ (1000 IU ml⁻¹) at various times points. Protein expression was measured by western blot analysis for (A) phosphorylated IKKα/β and (B) IκB. * P < 0.05 compared to untreated cells, ** P < 0.01 compared to untreated cells, *** P < 0.001 compared to untreated cells, # P < 0.05 compared to untreated cells, ## P < 0.01 compared to untreated cells, ### P < 0.001 compared to untreated cells.

**Fig. 9.** IFNγ-induced NF-κB-associated signaling pathway is sensitive to GC effects. A549 cells were treated for 15 or 30 min with IFNγ (1000 IU ml⁻¹) in the presence or absence of FP (100 nM) added 1 hr before. Protein expression of (A) phosphorylated IKKα/β and (B) IκB was measured by western blot analysis. (A) ** P < 0.05 compared to untreated cells, ## P < 0.01
compared to cells treated with IFNγ alone; (B) δ P < 0.05 compared to untreated cells, ** P < 0.01 compared to cells treated with IFNγ alone.

**Fig. 10. Silencing STAT1 reduces the expression of steroid insensitive IIGs, and restores GC responsiveness.** A549 cells were transfected with siRNA-STAT1 (250 nM) then treated with IFNγ (1000 IU ml⁻¹) 8 hr in the presence or absence of FP (100 nM) added 2 hr after. mRNA expression was examined using real time PCR analysis for steroid insensitive IIGs (A) CXCL11 and (B) IFIT2. Real time PCR results were normalized to β actin and expressed as percent of IFNγ induction. (C) A549 cells were transfected with siRNA-STAT1 (250 nM) for 48 hr then treated with IFNγ for 24 hr in the presence or absence of FP (100 nM) added 2 hr after. The secretion of CXCL11 in cells’ supernatants was examined by ELISA. # P <0.05, ## P <0.01, and ### P <0.001 compared to siControl cells treated with IFNγ alone.

**Fig. 11. IFNγ-induced STAT1 phosphorylation at Y701 residue is significantly higher in epithelial cells from patients with COPD than in epithelial cells from control patients.** (A) Epithelial cells from healthy and COPD patients cultured in ALI were treated with IFNγ (1000 IU ml⁻¹) for 8 hr in the presence or absence of FP (100 nM) added 2 hr after. IFNγ-induced STAT1 phosphorylation at Y701 residue was also insensitive to FP treatment. *** P <0.001 compared to healthy patients. (B) H&E staining of epithelial cells from a healthy patient cultured at ALI.
**Figure 1**

**A**
- **p-JAK1**
- **t-JAK1**
- **α actinin**

**Fold increase over basal**

- **FP (100 nM)**: 1.0, 2.0, 5.0, 6.0
- **IFNγ (1000 IU/ml)**: 1.0, 1.0, 3.0, 4.0
- **+**
- **NS**

**B**
- **p-JAK2**
- **t-JAK2**
- **α actinin**

**Fold increase over basal**

- **FP (100 nM)**: 1.0, 2.0, 5.0, 6.0
- **IFNγ (1000 IU/ml)**: 1.0, 2.0, 3.0, 4.0
- **+**
- **NS**

**C**
- **p-STAT1 (Y701)**
- **t-STAT1**
- **GAPDH**

**Fold increase over basal**

- **FP (100 nM)**: 1.0, 2.0, 3.0, 4.0
- **IFNγ (1000 IU/ml)**: 1.0, 2.0, 3.0, 4.0
- **IFNβ (1000 IU/ml)**: 1.0, 2.0, 3.0, 4.0
- **+**
- **#**

**D**
- **p-STAT1 (S727)**
- **t-STAT1**
- **GAPDH**

**Fold increase over basal**

- **FP (100 nM)**: 1.0, 2.0, 3.0, 4.0
- **IFNγ (1000 IU/ml)**: 1.0, 2.0, 3.0, 4.0
- **IFNβ (1000 IU/ml)**: 1.0, 2.0, 3.0, 4.0
- **/+**
- **#**
**Figure 2**

**A**

**Nuclear fraction**

<table>
<thead>
<tr>
<th>Protein</th>
<th>kDa</th>
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<tbody>
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<td>p-STAT (Y701)</td>
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<tr>
<td>t-STAT</td>
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</tr>
<tr>
<td>Lamin A/C</td>
<td>-64</td>
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**Fold increase over basal**

<table>
<thead>
<tr>
<th>Condition</th>
<th>FP (100 nM)</th>
<th>IFNγ (1000 IU/ml)</th>
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</thead>
<tbody>
<tr>
<td>Basal FP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Basal IFNγ</td>
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<td>+</td>
</tr>
<tr>
<td>FP +</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IFNγ + FP</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*NS* = Not significant

*** = Significant at p < 0.001

**B**

**p - STAT1 Y701**

**Basal**

**IFNγ**

**FP**

**IFNγ + FP**

**Figure 2**
Figure 3

A: GAS-luciferase reporter activity

<table>
<thead>
<tr>
<th>FP (100 nM)</th>
<th>IFNγ (1000 IU/ml)</th>
<th>Fold increase over basal</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>NS</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>**</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>***</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>NS</td>
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</table>

B: GAS-luciferase reporter activity

<table>
<thead>
<tr>
<th>FP (100 nM)</th>
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<th>Fold increase over basal</th>
</tr>
</thead>
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<tr>
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<td>+</td>
<td>2</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>NS</td>
</tr>
</tbody>
</table>
Figure 4
Figure 5
Figure 6

A

Percent of IFNγ induction

B

Percent of IFNγ induction

C

Percent of IFNγ induction

D

Percent of IFNγ induction

MTT assay (% of control)
**Figure 7**

(A) GAS-luc reporter activity

(B) Protein expression levels of p-STAT1 (Y701) and t-STAT1

(C) Protein expression levels of p-STAT1 (S727) and t-STAT1
Figure 8
Figure 9
Figure 10

Panel A: CXCL11

Panel B: IFIT2

Panel C: CXCL11 (pg/ml)

Graphs showing the percent of IFNγ induction and CXCL11 levels under different conditions:
- FP
- IFNγ
- siControl
- siSTAT1

Significance levels indicated:
- # for 36%
- ## for 62%
**Figure 11**

A. Western blot analysis showing the expression levels of p-STAT1 (Y701), t-STAT1, and GAPDH under different conditions. The chart indicates the fold increase over basal levels.

B. Microscopic image of tissue sections stained with hematoxylin and eosin (H&E).